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Switching Gears for an Influenza Pandemic: Validation of a Duplex Reverse Transcriptase PCR Assay for Simultaneous Detection and Confirmatory Identification of Pandemic (H1N1) 2009 Influenza Virus▽

Jason J. LeBlanc,[1]* Yan Li,[3] Nathalie Bastien,[3] Kevin R. Forward,[1,2] Ross J. Davidson,[1,2] and Todd F. Hatchette[1,2]

Department of Pathology and Laboratory Medicine, Queen Elizabeth II Health Science Center, Halifax, Nova Scotia, Canada[1]*; Department of Pathology, Dalhousie University, Halifax, Nova Scotia, Canada*[2]*; and National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada*[3]

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Rapid methods for the detection and confirmatory identification of pandemic influenza A virus (also known as pandemic [H1N1] 2009) are of utmost importance. In this study, a conventional reverse transcriptase PCR (RT-PCR) assay for the detection of influenza A virus and the hemagglutinin of swine lineage H1 (swH1) was designed, optimized, and validated. Nucleic acids were extracted from 198 consecutive nasopharyngeal, nasal, or throat swab specimens collected early in the outbreak (127 negative specimens, 66 specimens with pandemic [H1N1] 2009 influenza virus, 3 specimens with seasonal [H1N1] influenza A virus, and 2 specimens with seasonal [H3N2] influenza A virus). The performance characteristics of the duplex RT-PCR assay were assessed and compared to those of various detection methods: a monoplex RT-PCR assay at the National Microbiology Laboratory, a real-time RT-PCR assay using a Centers for Disease Control and Prevention protocol, an in-house multiplex RT-PCR assay (targeting influenza A virus, influenza B virus, and respiratory syncytial virus), and a rapid antigen test (the Binax Now Influenza A & B assay). The sensitivity of the duplex RT-PCR assay for influenza A virus detection was 97.2%, whereas the sensitivities were 74.6%, 71.8%, 47.8%, and 12.7% for the other four assays, respectively. The duplex RT-PCR assay was also able to identify swH1 in 94% of the cases, thereby reducing the number of specimens forwarded to reference laboratories for confirmatory identification. Only a limited number of specimens that contained influenza A virus had amounts of virus that fell below the limit of detection of the assay with the swH1 primers. Overall, the duplex RT-PCR assay is a reliable method for the simultaneous detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus and would be particularly attractive to laboratories without real-time RT-PCR capabilities.

Pigs and humans have many similarities with respect to influenza viruses. Both have well-established, distinct, and stable lineages of influenza A virus that cause periodic epidemics associated with morbidity and mortality (21). The swine influenza A viruses currently circulating in North America are triple reassortants that have components of avian, human, and swine origin (19). For pandemic (H1N1) 2009 influenza virus, the PB2 and PA polymerase components are derived from avian influenza virus lineages; PB1 is of human influenza virus origin; and the genes encoding hemagglutinin (HA), neuraminidase, nucleoprotein, matrix protein (M), and nonstructural protein are of swine lineages of influenza A virus (16). Sporadic cases of human infection with triple-reassortant swine influenza viruses were previously documented; however, until recently, human-to-human transmission was not sustained (6, 16, 19). In March 2009, Mexico reported clusters of a respiratory disease that was subsequently identified as pandemic (H1N1) 2009 influenza virus. As of 30 August 2009, over 250,000 cases and 2,837 deaths had been documented worldwide (23). This led the WHO to increase the pandemic alert to phase 6 and declare an international public health emergency.

Rapid diagnosis by molecular methods, such as reverse transcriptase PCR (RT-PCR) assays, is the cornerstone of planning for a pandemic. As outlined in the Canadian Pandemic Influenza Plan (18), provincial public health or designated laboratories should have the capacity to identify and subtype influenza viruses using molecular methods. The high sensitivity and specificity of RT-PCR compared to those of conventional detection methods prompted many laboratories to implement RT-PCR for the detection of influenza viruses. With increasing rates of antiviral resistance in circulating seasonal human influenza A virus strains (H1N1 and H3N2), RT-PCR is now being used for influenza A virus subtyping in order to facilitate the clinical management of patients (9). Influenza A viruses that cannot be subtyped must be forwarded to reference laboratories to rule out the presence of a novel influenza virus strain (3, 18). This was the scenario that played out in the Capital District Health Authority (CDHA) microbiology laboratory in Hali-

* Corresponding author. Mailing address: Division of Microbiology, Department of Pathology and Laboratory Medicine, Queen Elizabeth II Health Sciences Center, 5788 University Avenue, Room 315, MacKenzie Building, Halifax, Nova Scotia B3H 1V8, Canada. Phone: (902) 473-7716. Fax: (902) 473-6432. E-mail: jason.leblanc@cdha.nshealth.ca.

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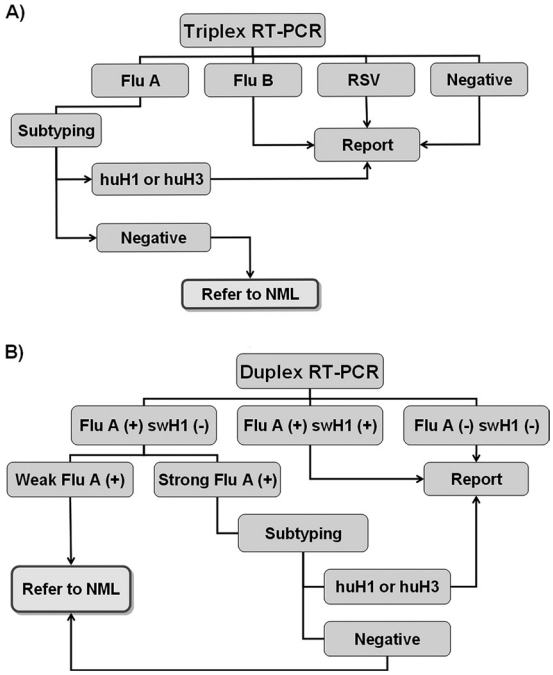

FIG. 1. Testing algorithms for detection of influenza A viruses. (A) Prior to the pandemic (H1N1) 2009 influenza virus outbreak, routine testing for influenza A virus (Flu A), influenza B virus (Flu B), and RSV was performed in Nova Scotia. Followed the detection of influenza A virus, huH1 and huH3 subtyping was performed. Specimens that could not be subtyped were forwarded to NML for sequence analysis. (B) Following the confirmation of cases of pandemic (H1N1) 2009 influenza virus, the testing strategies focused on screening for influenza A virus and swH1. Specimens that were weakly positive for influenza A virus or that failed subtyping were referred to NML.

fax, Nova Scotia, Canada, and many other laboratories across North America.

On 24 April 2009, the CDHA microbiology laboratory received specimens from five symptomatic individuals with epidemiological links to Mexico (4). Three of the five specimens were found to contain influenza A virus, but the isolates were nontypeable with primers targeting human hemagglutinins H1 (huH1) and H3 (huH3) from seasonal influenza A viruses. These three cases and an additional case were confirmed to be pandemic (H1N1) influenza virus by the National Microbiology Laboratory (NML) by RT-PCR and sequencing of the M gene (4). While these methods enabled the detection of the first Canadian cases, more timely methods were necessary to help guide public health management. In fact, following confirmatory identification of this novel influenza A virus in Nova Scotia, there was a dramatic increase in the number of respiratory specimens submitted for influenza virus RT-PCR. Initial strategies based on screening for influenza A virus followed by subtyping extended the turnaround times and put tremendous stress on both human resources and the available reagents. As such, our traditional testing algorithm had to be quickly revised to accommodate this surge (Fig. 1). With primers designed by NML targeting the HA from H1 lineages of swine influenza A virus (swH1) and a second primer pair targeting influenza A virus (5), we validated a duplex RT-PCR assay for the simultaneous detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus.

MATERIALS AND METHODS

Specimen collection. Nasal, nasopharyngeal, or throat swab specimens were collected from individuals during the recent outbreak in Nova Scotia (4). The swabs were placed in universal transport medium (Copan Diagnostics, Corona, CA) and maintained at 4°C until they were tested or were aliquoted and stored at −80°C for long-term storage. Validation of the duplex RT-PCR assay was performed with 198 consecutive specimens collected between 26 April and 28 April 2009. An additional 50 positive specimens and 50 negative specimens collected between 23 April and 8 May 2009 were used as part of the retrospective analysis; specimens processed during the validation period were excluded. The analytical specificity of the assay was evaluated with a panel of archived viruses (Table 1). These included various human, avian, and swine influenza A viruses; influenza B virus; parainfluenza virus (PIV) type 1 (PIV-1); PIV-2; and PIV-3; respiratory syncytial virus (RSV); herpes simplex virus (HSV) type 1 (HSV-1) and HSV-2; cytomegalovirus (CMV); enterovirus; mumps virus (genotype G); and adenovirus.

TABLE 1. Virus isolates used for the specificity panel

Virus	Description	Duplex RT-PCR assay result for:	
		Influenza A virus	swH1
Influenza A virus	A/Canada-NS/RV 1535/2009 (H1N1)v	+	+
	A/Canada-NS/RV 1536/2009 (H1N1)v	+	+
	A/Canada-NS/RV 1538/2009 (H1N1)v	+	+
	A/Swine/Minnesota/3068ZT/98 (H1N1)	+	+
	A/Solomon Islands/03/06 (H1N1)	+	−
	A/New Caledonia/20/99 (H1N1)	+	−
	A/Wisconsin/67/05 (H3N2)	+	−
	A/Swine/Texas/4199-2/98 (H3N2)	+	−
	A/Duck/Czech/56 (H4N6)	+	−
	A/Turkey/Wisconsin/68 (H5N9)	+	−
	A/Turkey/Massachusetts/3740/65 (H6N2)	+	−
	A/Shearwater/Australia/72 (H6N5)	+	−
	A/Turkey/Oregon/71 (H7N3)	+	−
	A/Turkey/Ontario/3778/68 (H8N4)	+	−
	A/Turkey/Wisconsin/1/66 (H9N2)	+	−
	A/Quail/Italy/1117/65 (H10N8)	+	−
	A/Duck/England/56 (H11N6)	+	−
	A/Duck/Wisconsin/480/79 (H12N6)	+	−
	A/Gull/Maryland/704/77 (H13N6)	+	−
Influenza B virus	B/Malaysia/2506/04	−	−
	B/Florida/07/07	−	−
RSV	Clinical isolate	−	−
PIV-1	Clinical isolate	−	−
PIV-2	Clinical isolate	−	−
PIV-3	Clinical isolate	−	−
Enterovirus	Clinical isolate	−	−
Mumps virus	Clinical isolate	−	−
Adenovirus	Clinical isolate	−	−
HSV-1	Clinical isolate	−	−
HSV-2	Clinical isolate	−	−
CMV	Clinical isolate	−	−

TABLE 2. Oligonucleotides and probes used in this study

Virus	Location	Name	Sequence (5′–3′)	Reference or source
Influenza A virus (M gene)	17–37[a]	FluA-M52C	CTTCTAACCGAGGTCGAAACG	5
	238–261[a]	FluA-M253R	AGGGCATTTTGGACAAAKCGTCTA	5
	331–351[a]	swH1M351R	TCCTTGGCCCCATGGAAYGTT	This study
	151–177[a]	InfA Forward[f]	GACCRATCCTGTCACCTCTGAC	2
	211–234[a]	InfA Reverse[f]	AGGGCATTYTGGACAAAKCGTCTA	2
	238–261[a]	InfA probe[f]	TGCAGTCCTCGCTCACTGGGCACG	2
	11–31[a]	FWISM	GGCGGATCCATGAGCCTTCTAACCGAGGTC	This study
	972–992[a]	RWISM	GGCCTCGAGTTACTCCAACTCTATGCTGAC	This study
Influenza A virus (HA gene)	107–127[b]	SwH1F	CAGACACTGTAGACACAGTAC	This study
	602–623[b]	SwH1R	CTAGTAGATGGATGGTGAATGC	This study
	920–942[b]	SW H1 Forward[f]	GTGCTATAAACACCAGCCTYCCA	2
	1010–1035[b]	SW H1 Reverse[f]	CGGGATATTCCTTAATCCTGTRGC	2
	946–975[b]	SW H1 probe[f]	CAGAATATACATCCRGTCACAATTGGARAA	2
	63–86[b]	H1-1	GATGCAGACACAATATGTATAGG	25
	635–658[b]	H1-2	CICTACAGAGACATAAGCATTT	25
	248–269[b]	HA1-230-F	GGATCTTAGGAAACCCAGAATG	This study
	756–775[b]	HA1 757-R	GTTCCAGCAGAGTCCACTAG	This study
	100–120[c]	H3ha100f	CATGCAGTACCAAACGGAACG	This study
	394–415[c]	H3ha415r	CATTGTTAAACTCCAGTGTGCC	This study
	144–165[c]	H3-1	TCAGATTGAAGTGACTAATGCT	25
	1100–1120[c]	H3-2	AATTTTGATGCCTGAAACCGT	25
Influenza B virus (M gene)	90–109[d]	FluB-B/MP	TTACACTGTTGGTTCGGTGG	13
	594–613[d]	FluB/MP-1R	GGCAGTTTTTGGACGTCTTC	13
RSV (F gene)	1111–1133[e]	HRSVMPFO2	AACAGTTTAACATTACCAAGTGA	14
	1468–1490[e]	HRSVMPRW2	TCATTGACTTGAGATATTGATGC	14

[a] Oligonucleotides are numbered as aligned to GenBank accession number FJ998210 for sequences encoding the matrix protein.
[b] Oligonucleotides are numbered as aligned to GenBank accession number FJ998207 for hemagglutinin H1 of influenza A/Canada-NS/RV1535/2009 (H1N1) virus.
[c] Oligonucleotides are numbered as aligned to GenBank accession number EU399751 for hemagglutinin H3 of influenza A/Ontario/1252/2007 (H3N2) virus.
[d] Oligonucleotides are numbered as aligned to GenBank accession number CY018486 for the M gene of influenza B/Canada/1688/2000 virus.
[e] Oligonucleotides are numbered as aligned to GenBank accession number AF013254 for the fusion gene of human RSV.
[f] Primers and probes sequences as well as the protocol for the real-time RT-PCR were provided by the CDC (2). The TaqMan probe was labeled at the 5′ end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher BlackHole Quencher 1 (BHQ1) (BioSearch Technologies, Inc., Novato, CA).

Influenza A virus rapid antigen testing. Rapid antigen testing was performed by use of a lateral flow assay kit, the Binax Now Influenza A & B kit (Inverness Medical, Ottawa, Ontario, Canada). One hundred microliters was processed in accordance with the manufacturer's instructions, and the plates were visually inspected after 15 min.

Nucleic acid extraction. For the monoplex influenza A virus or the swH1 RT-PCR assay (performed at NML), viral RNA was extracted from 265 μl of specimen with a BioRobot MDx viral kit (Qiagen Inc., Mississauga, Ontario, Canada) on a BioRobot MDx apparatus (Qiagen Inc.), and the RNA was eluted in a final volume of 100 μl. All other nucleic acid extractions were performed at the CDHA microbiology laboratory with a MagNA Pure LC instrument (Roche Diagnostics, Branchburg, NJ) and a total nucleic acid isolation kit (Roche Diagnostics). One hundred forty microliters of specimen was extracted, as recommended in the manufacturer's instructions, and nucleic acids were eluted in a final volume of 60 μl. Five microliters served as the template in all RT-PCR assays.

Conventional RT-PCR assay. Conventional RT-PCR assays (monoplex, duplex, triplex, and HA subtyping assays) were performed with a Qiagen one-step RT-PCR kit. Oligonucleotides (Table 2) were synthesized by Sigma Genosys (Oakville, Ontario, Canada), with the exception that the swH1-specific primer pair was synthesized by NML. RT-PCR amplifications were performed in 48- or 96-well plates on a DNA engine dyad thermocycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). The amplicons were resolved by 1% agarose gel electrophoresis with ethidium bromide staining.

Monoplex RT-PCR assay targeting influenza A virus or swH1. Viral RNA was amplified in a one-step RT-PCR (Qiagen Inc.), according to the manufacturer's recommendations. Briefly, 5 μl of RNA was added to a 50-μl RT-PCR mixture containing 2 μl One-Step RT-PCR enzyme mixture, 1× One-Step RT-PCR buffer, 10 μl of Q solution, 400 μM deoxynucleoside triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), and 0.6 μM of each primer. Primers specific for influenza A virus, primers FluA-M52C and FluA-M253R, have been described previously (5). Primers targeting the HA of swH1, primers SwFluAH1F and SwFluAH1R, were developed by NML on the basis of HA sequence data from influenza A/California/04/2009 (H1N1) virus (GenBank accession number FJ966082) obtained from the Global Initiative on Sharing Avian Influenza Data. The thermocycling conditions were those recommended by NML: reverse transcription at 50°C for 30 min; activation of the HotStart DNA polymerase at 95°C for 15 min; and then 40 cycles of denaturation at 94°C for 30 s, annealing at 59°C (influenza A virus) or 50°C (swH1) for 30 s, and extension at 72°C for 1 min, followed by a final extension of 10 min at 72°C. The expected product sizes were 244 bp for influenza A virus and 517 bp for swH1.

Influenza A virus and swH1 duplex RT-PCR assay. For the influenza A virus and swH1 duplex RT-PCR assay, the 50-μl reaction mixtures contained 5 μl of template, 2 μl of One-Step RT-PCR enzyme mixture, 1× One-Step RT-PCR buffer, 10 μl of Q-solution, 1 mM dNTPs, 5 U of RNaseOUT recombinant RNase inhibitor (Invitrogen Canada Inc., Burlington, Ontario, Canada), and 1 μM of each primer pair (primers FluA-M52C and FluA-M253R for influenza A virus [5] and primers SwFluAH1F and SwFluAH1R for swH1). The thermocycling conditions were as follows: reverse transcription at 50°C for 30 min; activation of the HotStart DNA polymerase at 95°C for 15 min; and then 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension of 10 min at 72°C. During optimization, annealing temperatures of 50°C and 60°C were also evaluated. For optimization of the duplex assay, a gradient RT-PCR assay was performed on a DNA engine dyad thermocycler (Bio-Rad Laboratories Ltd.) by using annealing temperatures ranging from 50 to 60°C.

For the nested PCRs, the 50-μl reaction mixtures contained 2 μl of amplicon, 1× buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs, 2.5 U of *Taq* DNA polymerase (Invitrogen Canada Inc.), and 1 μM of each primer. The thermocycling conditions were as follows: initial activation at 95°C for 5 min; then 25 cycles of denaturation at 95°C for 30 s, annealing at 50 or 55°C for 30 s, and extension 72°C for 60s; and a final elongation at 72°C for 7 min.

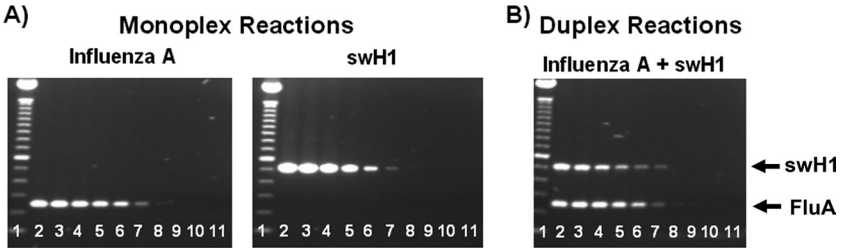

FIG. 2. The monoplex and duplex RT-PCR assays have equivalent LODs. Tenfold serial dilutions of pandemic (H1N1) 2009 influenza virus RNA were subjected to the monoplex reaction (influenza A virus or swH1) or the duplex reaction (influenza A virus and swH1). Lanes 1, 100-bp ladder; lanes 2 to 10, dilutions ranging from 10^0 to 10^{-8}; lane 11, reagent control.

Influenza A virus, influenza B virus, and RSV triplex RT-PCR assay. For the influenza A virus, influenza B virus and RSV triplex RT-PCR assay (1), the 50-μl reaction mixtures contained 5 μl of template, 2 μl of One-Step RT-PCR enzyme mix, 1× One-Step RT-PCR buffer, 1 mM dNTPs, 20 U RNaseOUT (Invitrogen Canada Inc.), and 1 μM of each primer pair (primers FluA-M52C and FluA-M253R for influenza A virus [5], primers FluB-B/MP and FluB/MP-1R for influenza B virus [13], and primers HRSVMPFOR1 and HRSVMPRW2 for RSV [14]). Thermocycling was performed as follows: reverse transcription at 50°C for 30 min; initial activation at 95°C for 15 min; and then 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension 72°C for 60 s, followed by a final elongation at 72°C for 7 min. The expected amplicon sizes were 244 bp, 380 bp, and 525 bp for influenza A virus, influenza B virus, and RSV, respectively.

Human influenza A virus subtyping. For huH1 and huH3 subtyping, the 50-μl reaction mixtures contained 5 μl of template, 2 μl of One-Step RT-PCR enzyme mix, 1× One-Step RT-PCR buffer, 1 mM dNTPs, 20U RNaseOUT, and 1 μM of each primer pair (Table 2) (primers HA1-230-F and HA1-757-R for huH1 and primers H3ha100f and H3ha415r for huH3). Thermocycling was performed as follows: reverse transcription at 50°C for 30 min; initial activation at 95°C for 15 min; and 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension 72°C for 60 s, followed by a final elongation at 72°C for 7 min. The expected amplicon sizes were 611 bp and 976 bp, respectively. Alternative primer pairs (primers H1-1 and H1-2 for huH1 and primers H3-1 and H3-2 for huH3) were also used when subtyping by the conventional assay failed (25). The expected product sizes were 529 bp and 316 bp, respectively.

Real-time RT-PCR. The detection of influenza A virus or swH1 by real-time RT-PCR was performed independently on a LightCycler (version 2.0) instrument (Roche Diagnostics) by using the thermocycling and reaction conditions described by the Centers for Disease Control and Prevention (CDC; Atlanta, GA) (2). A one-step RT-PCR was performed by using a Qiagen QuantiTect Multiplex NoROX RT-PCR kit (Qiagen Inc.) in 20-μl reaction mixtures consisting of 1× master mix, 0.2 μl QuantiTect multiplex NoRox enzyme mixture, 20 U RNaseOUT, 400 nM of primers (primers InfA Forward and InfA Reverse for influenza A virus; primers SW H1 Forward and SW H1 Reverse for swH1), and 200 nM of FluA probe (InfA probe for influenza A virus and SW H1 probe for swH1) (Table 2).

The viral copy number was estimated in relation to the numbers on a standard curve generated by using a linearized plasmid harboring the M gene of influenza A/Wisconsin/67/2005 virus. Briefly, the amplicon generated from PCR amplification with primer pair FWISM and FWISM was subcloned into the XhoI and BamHI restriction sites of similarly digested pBlueScript II KS− (Stratagene, La Jolla, CA). Following electroporation into *Escherichia coli* XL10 Gold (Stratagene), confirmation that the transformants were ampicillin resistant was performed by PCR. Plasmid was extracted by using a QIAprep Spin miniprep kit (Qiagen Inc.), digested with BamHI, and subjected to agarose gel electrophoresis. Following purification with a QIAquick gel extraction kit (Qiagen Inc.), linearized plasmid was quantified by spectrophotometry. Tenfold serial dilutions of linearized plasmid were used as the template for the real-time RT-PCR. An inverse linear relationship ($y = -3.10x + 38.95$; $R^2 = 0.9979$) was generated by plotting the crossing-point values against the plasmid concentration.

DNA sequencing. The M-gene amplicons from specimens positive for influenza A virus by the duplex RT-PCR assay were purified with a QIAquick PCR purification kit (Qiagen Inc.) and were subjected to sequence analysis with primers FluA-M52C and FluA-M253R (5). To ensure that discrepant results were not due to amplicon contamination, additional specimens found to be positive by the duplex RT-PCR assay were subjected to a second RT-PCR assay and sequencing reaction with primer FluA-M52C and a second primer (primer swH1M351R) whose sequence was located downstream of the original targeted region (Table 2). Sequencing was conducted on an ABI 3100 sequencer (Applied BioSystems, Streetsville, Ontario, Canada) at the DNA Core Facility at NML or by using BigDye Terminator chemistry on an ABI 3130×L DNA sequencer (Applied BioSystems) at York University (Toronto, Ontario, Canada). Sequence analysis was performed with Lasergene (version 7.1) sequence analysis software (DNAStar, Madison, WI), and the consensus sequences (from the forward and reverse sequencing reactions) were compared to reference data available in the GenBank database by using BLAST analysis.

Statistics. Since the optimal RT-PCR assay for the detection of pandemic (H1N1) 2009 influenza virus is unknown, a modified "gold standard" was used to assess the clinical performance of all RT-PCR assays when a positive case was defined by concordant results between at least two RT-PCRs targeting different genomic regions and subsequent sequence analysis to ensure the specificities of the primers. The performance of each method was compared to this modified gold standard to determine the sensitivities, specificities, positive predictive values, and negative predictive values. The 95% confidence intervals were calculated for each value. Chi-square and two-tailed Fisher's exact tests were used, and a P value of <0.05 was considered statistically significant.

RESULTS

Monoplex versus duplex RT-PCR assays. Tenfold serial dilutions of pandemic (H1N1) 2009 influenza virus RNA were prepared to compare the analytical sensitivity of the monoplex RT-PCR assay (influenza A virus or swH1) and the duplex RT-PCR assay (influenza A virus and swH1). In three independent experiments, both assays demonstrated similar limits of detection (LODs). For influenza A virus, the LOD was estimated to be 2 to 20 copies per reaction (10^{-6} or 10^{-7} dilution), whereas for swH1, the LOD was approximately 20 to 200 copies per reaction (10^{-5} and 10^{-6} dilutions) (Fig. 2). The increased sensitivity for the detection of influenza A virus compared with that for the detection of swH1 suggests that confirmatory identification would be required with specimens containing low viral loads (Fig. 1B). No additional benefit was afforded by use of the nested PCR (except for an increased amplicon quantity). On the other hand, the gradient RT-PCR assay, which varied the annealing temperature to values spanning 50°C to 60°C (Fig. 3), demonstrated that at 50°C, the amplification of swH1 may not be optimal. When 10-fold serial dilutions of pandemic (H1N1) 2009 influenza virus RNA were tested at annealing temperatures of 50°C, 55°C, and 60°C, the detection of swH1 was optimal at 55°C (Fig. 3). At this temperature, the detection of swH1 was approximately 10-fold more sensitive than the sensitivity obtained by the use of the recommended temperature of 50°C. Whatever the annealing temperature used, no differences were observed for influenza A virus.

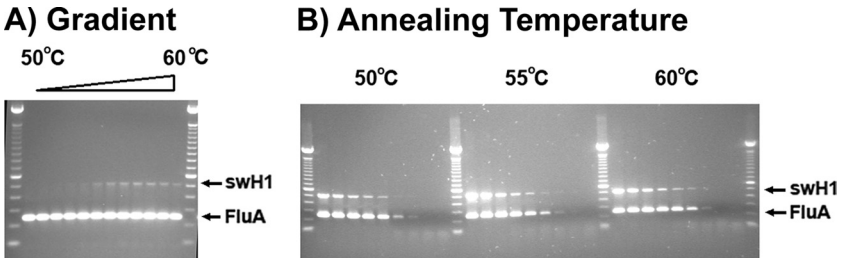

FIG. 3. Optimization of the duplex RT-PCR assay. (A) By using RNA concentrations 10-fold less than the LOD of swH1, a gradient RT-PCR was performed at annealing temperatures spanning 50°C to 60°C. Estimates of the temperatures achieved are as follows (the numbers for the unnumbered lanes apply to the lanes from left to right, respectively): 50.0°C (lane 2), 50.3°C (lane 3), 50.9°C (lane 4), 51.7°C (lane 5), 52.8°C (lane 6), 54.3°C (lane 7), 56.0°C (lane 8), 57.4°C (lane 9), 58.5°C (lane 10), 59.3°C (lane 11), 59.8°C (lane 12), and 60.0°C (lane 13). A 100-bp ladder is found in lanes 1 and 14. (B) The duplex RT-PCR assay was performed using annealing temperatures of 50°C, 55°C, and 60°C.

Analytical specificity. Identical primers targeting a highly conserved region of the influenza A virus matrix gene are present in the monoplex and the duplex RT-PCR assays (5). While the specificity of the influenza A virus-specific primers has been well documented (1, 5), the influence of the swH1-specific primers was unknown. The specificity of the duplex RT-PCR assay was assessed by using a panel of archived viruses (Table 1). The influenza A virus amplicon was observed for all influenza A virus strains tested, whereas the swH1 amplicon was observed only with pandemic (H1N1) 2009 influenza virus isolates or A/Swine/Minnesota/3068ZT/98 (H1N1) virus. No amplification was observed when nucleic acids extracted from others viruses (RSV, HSV-1, HSV-2, CMV, PIV-1, PIV-2, PIV-3, enterovirus, mumps virus, adenovirus, and influenza B virus) were used.

Analytical sensitivity. The analytical sensitivities of the duplex, triplex, and real-time RT-PCR assays were evaluated with nucleic acids extracted from 10-fold dilutions of a pandemic (H1N1) 2009 influenza A virus isolate, the A/Canada-NS/RV1535/2009 (H1N1) virus (Fig. 4). As seen in Fig. 2, the LOD of influenza A virus in the duplex assay was approximately two copies per reaction (10^{-5} dilution), whereas the LOD for swH1 was approximately 20 to 200 copies per reaction (10^{-3} or 10^{-4} dilution) (Fig. 4A). The sensitivity of the real-time RT-PCR for influenza A virus was approximately two copies per reaction (10^{-5} dilution; Fig. 4C); however, at this concentration, a positive signal was obtained in only two of five reactions. The duplex RT-PCR assay obtained positive signals in all five reactions, suggesting that the real-time RT-PCR assay may be less sensitive. As for the triplex RT-PCR assay, a lower LOD

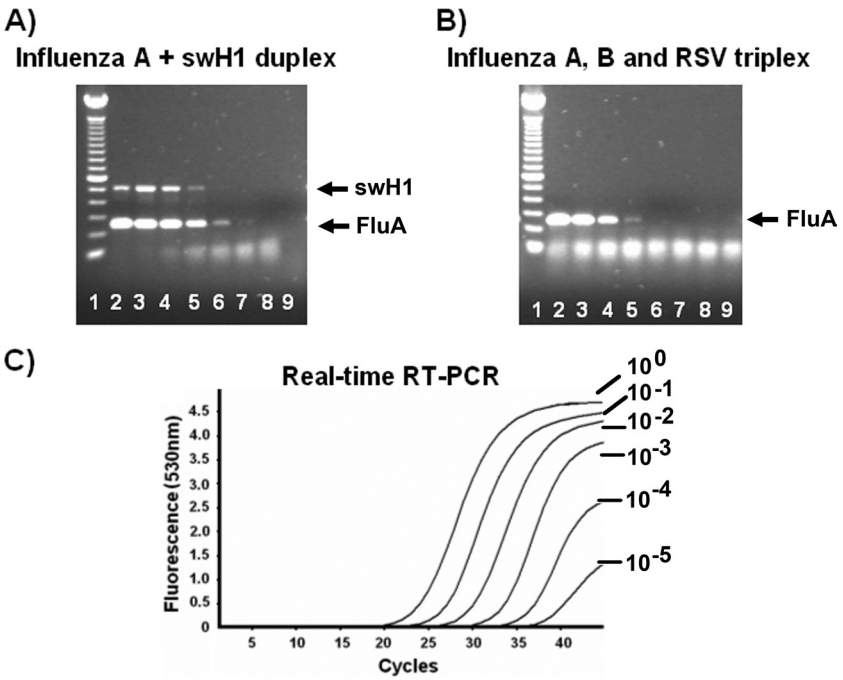

FIG. 4. Endpoint analysis of three RT-PCR assays targeting influenza A virus. Nucleic acids extracted from 10-fold serial dilutions of pandemic (H1N1) 2009 influenza virus were subjected to the influenza A virus and swH1 duplex RT-PCR assay (A); the influenza A virus, influenza B virus, and RSV triplex RT-PCR assay (B); and a real-time RT-PCR assay targeting influenza A virus. Lanes 1, 100-bp ladder; lanes 2 to 7, dilutions ranging from 10^0 to 10^{-5}; lanes 8, reagent control.

was observed at approximately 200 copies per reaction (10^{-3} dilution) (Fig. 4B). The lack of sensitivity of the triplex RT-PCR assay was not unique to the pandemic (H1N1) 2009 influenza virus. When nucleic acids extracted from 10-fold serial dilutions of seasonal influenza A viruses (H1N1 and H3N2) were used, the triplex RT-PCR assay was 10- to 100-fold less sensitive than the duplex RT-PCR assay (data not shown).

Clinical performance. The RT-PCR assays and the rapid antigen test were assessed by analyzing 198 consecutive specimens collected early during the outbreak in Nova Scotia. Of 71 specimens confirmed to contain influenza A viruses by sequencing, the viruses in 66 were identified as pandemic (H1N1) 2009 influenza virus, the viruses in 3 were identified as seasonal H1N1 virus, and the viruses in 2 were identified as seasonal H3N2 virus. The sensitivity of the duplex RT-PCR assay for influenza A virus detection was 97.2% (69/71 specimens), whereas all other assays were significantly ($P < 0.001$) less sensitive: 74.6% (53/71) for the influenza A virus monoplex RT-PCR assay, 71.8% (51/71) for the real-time RT-PCR assay, 47.8% (34/71) for the triplex RT-PCR assay, and 12.7% (9/71) for rapid antigen testing (Table 3). With a prevalence of 36% (71/198), the negative predictive values were 98.4% for the duplex RT-PCR assay, 87.6% for the influenza A virus monoplex RT-PCR assay, 86.4% for the real-time RT-PCR assay, 77.4% for the triplex RT-PCR assay, and 67.2% for rapid antigen testing (Table 3). No false-positive results were observed, giving all assays positive predictive values of 100%.

Of 71 influenza A viruses identified in this study, 66 were confirmed to be pandemic (H1N1) 2009 influenza virus. Of the RT-PCR assays able to identify swH1 (the monoplex, duplex, and real-time RT-PCR assays), the sensitivities differed (Table 3). The duplex RT-PCR assay identified swH1 in most cases and had a sensitivity of 94% (62/66). However, this high degree of sensitivity was observed only if the annealing temperature was set at 55°C. By using an annealing temperature of 50°C, the sensitivity for the detection of swH1 fell to 77% (51/66). Interestingly, a similar sensitivity (80% [51/66]) was observed by the swH1 monoplex RT-PCR assay, which was performed at an annealing temperature of 50°C. These data, along with the results presented in Fig. 3, suggest that the duplex RT-PCR assay should be performed at an annealing temperature of 55°C.

As described in the Materials and Methods, a positive pandemic (H1N1) 2009 influenza virus case was defined by concordant results between at least two RT-PCR assays targeting different genomic regions and subsequent sequence analysis to ensure the specificities of the primers. However, the duplex RT-PCR assay identified 18 additional influenza A virus isolates that were considered negative by the real-time influenza A virus RT-PCR assay. To ensure that the detection of the additional influenza A virus cases by the duplex RT-PCR assay could not be attributed to amplicon contamination, several strategies were undertaken. First, reextraction and a repeat duplex RT-PCR assay generated identical results, with two exceptions. The influenza A virus monoplex RT-PCR assay had previously identified two cases of pandemic (H1N1) 2009 influenza virus infection that were considered negative by all other assays, including the duplex RT-PCR assay; however, upon the repeat of the duplex RT-PCR assay, these two cases

TABLE 3. Performance characteristics of the various assays[a]

Assay	Influenza A virus				swH1			
	% Sn	% Sp	% PPV	% NPV	% Sn	% Sp	% PPV	% NPV
Duplex RT-PCR (55°C)[b]	97.2 (93.8–97.2)	100 (98.1–100)	100 (96.5–100)	98.4 (93.1–98.4)	93.9 (90.0–93.9)	100 (98.0–100)	100 (95.8–100)	97.1 (95.1–97.1)
Duplex RT-PCR (50°C)[b]	97.2 (93.8–97.2)	100 (98.1–100)	100 (96.5–100)	98.4 (93.1–98.4)	77.3 (72.6–77.3)	100 (97.7–100)	100 (94.0–100)	89.8 (87.7–89.8)
Monoplex RT-PCR[c]	74.6 (70.3–74.6)	100 (97.6–100)	100 (94.1–100)	87.6 (85.4–87.6)	80.3 (75.7–80.3)	100 (97.7–100)	100 (94.3–100)	91.0 (89.0–91.0)
Real-time RT-PCR[c]	71.8 (67.4–71.8)	100 (97.5–100)	100 (93.9–100)	86.4 (84.3–86.4)	90.9 (86.7–90.9)	100 (97.9–100)	100 (95.4–100)	95.7 (93.6–100)
Triplex RT-PCR	47.9 (43.4–47.9)	100 (97.5–100)	100 (90.6–100)	77.4 (75.5–77.4)				
Rapid antigen testing	12.7 (9.0–12.7)	100 (97.9–100)	100 (71.0–100)	67.2 (65.8–67.2)				

[a] The sensitivities (Sn), specificities (Sp), positive predictive values (PPV), and negative predictive values (NPV) were calculated by comparison to the results obtained with a modified gold standard (see Materials and Methods); 95% confidence intervals are indicated in parentheses. The results for influenza A virus are based on 71 confirmed cases (66 cases of pandemic [H1N1] 2009 influenza virus infection, 3 cases of seasonal [H1N1] influenza A virus infection, and 2 cases of seasonal [H3N2] influenza virus infection). The results for swH1 were calculated on the basis of the data for the 66 confirmed cases of pandemic (H1N1) 2009 influenza virus infection. A P value of <0.05 was considered statistically significant.

[b] The duplex RT-assay PCR was evaluated by using annealing temperatures of 50°C and 55°C.

[c] For the monoplex and real-time RT-PCRs, the detection of influenza A virus and swH1 was performed in independent reactions.

were influenza A positive (but swH1 negative). Second, the real-time swH1 RT-PCR assay confirmed the results for 91% (60/66) of the pandemic (H1N1) 2009 influenza virus cases (Table 3). All cases that were positive by the real-time RT-PCR assay were also positive by the duplex RT-PCR assay. Of note, 10 of the 60 swH1-positive specimens detected by the real-time RT-PCR assay had crossing-point values between 35 and 40, suggesting that these specimens had low viral loads. Finally, a second conventional RT-PCR assay which incorporated both a higher concentration of template RNA (obtained by using a larger amount of specimen eluted in the same volume) and primers designed outside the region targeted by the duplex RT-PCR assay was used. The amplicon was present in all samples with discrepant results analyzed, including those confirmed to be positive by the real-time swH1 RT-PCR assay. These amplicons were purified and subjected to sequencing of the M gene, which revealed 15 pandemic (H1N1) 2009 influenza viruses and 3 seasonal influenza A viruses (two H1N1 viruses and one H3N2 virus).

Retrospective analysis. To ensure that the duplex RT-PCR assay had an adequate performance following its implementation, 50 specimens negative for influenza A virus and 50 specimens positive for influenza A virus were subjected to both the duplex and the triplex RT-PCR assays. Of the 50 positive specimens, both the duplex and the triplex RT-PCR assays detected influenza A virus in 29 of them. By using sequencing and huH1 and huH3 subtyping, these isolates were identified as follows: 16 were pandemic (H1N1) 2009 influenza virus isolates, 7 were seasonal H1N1 virus isolates, and 6 were seasonal H3N2 virus isolates. All 16 pandemic (H1N1) 2009 viruses were swH1 positive by the duplex RT-PCR assay. It should be noted that 21 pandemic (H1N1) 2009 viruses were detected only by the duplex RT-PCR assay, suggesting that the triplex RT-PCR assay would have missed 42% of cases. While 18 of these 21 cases were found to be swH1 positive by the duplex RT-PCR assay, the results for the remaining three influenza A virus-positive specimens could not be resolved by subtyping (swH1, huH1, or huH3) or the nested PCR assay. By using sequence analysis following RT-PCR with primers designed to have sequences outside the original targeted region and real-time swH1 RT-PCR, these three were confirmed to be pandemic (H1N1) 2009 influenza virus.

Of interest, the results for the original five nasal swab specimens submitted to the CDHA microbiology laboratory (4) were evaluated within the retrospective data. Three were identified to contain influenza A virus by the triplex RT-PCR assay, four were identified to contain influenza A virus by the monoplex RT-PCR assay, and all five were identified to contain influenza A virus by the duplex RT-PCR assay. In addition, two of five specimens were swH1 positive by the duplex RT-PCR assay. The additional cases detected by the monoplex and duplex RT-PCR assays required sequence analysis, suggesting that these specimens contained low viral loads. All five were confirmed to contain pandemic (H1N1) 2009 influenza virus by sequence analysis.

DISCUSSION

The early detection of infected patients, the implementation of isolation measures, and contact tracing are imperative for the management of influenza virus infections. Rapid antigen tests can generate a result in 30 min or less (18, 23); unfortunately, these methods lack sensitivity (Table 3) compared to that of RT-PCR (7, 8, 10, 24) and should not be used to exclude the possibility of an influenza virus infection. Early in the pandemic (H1N1) 2009 influenza virus outbreak, the only available guidelines suggested that the identities of novel influenza viruses should be confirmed by viral culture and at least partial sequencing of the viral genome (3, 24). While sequencing is considered the gold standard for the confirmatory identification of novel influenza viruses, the use of this approach is impractical for most laboratories and poses problems for the routine detection of influenza viruses by RT-PCR (discussed later). Using sequence data from pandemic (H1N1) 2009 influenza virus, we designed primers targeting swH1 and validated the performance of a conventional RT-PCR assay that is capable of simultaneously detecting and confirming the identity of this novel influenza A virus.

The duplex RT-PCR assay was significantly more sensitive than all other assays for the detection of influenza A virus (Table 3), including the conventional triplex RT-PCR assay previously used in the CHDA microbiology laboratory (Fig. 4). This prompted a rapid modification of the influenza testing algorithm during the Nova Scotia outbreak (Fig. 1). Interestingly, the duplex RT-PCR assay was also more sensitive than the real-time influenza A virus RT-PCR designed by the CDC (2). The lower sensitivity of the real-time RT-PCR could partly be attributed to the protocol, which had not yet been optimized for use on the LightCycler platform. However, Poon et al. (17) also recently found that a real-time RT-PCR assay was less sensitive than a conventional RT-PCR assay for the detection of A/Swine/Hong Kong/PHK1578/03 virus. It remains to be determined whether the sensitivity of real-time influenza A virus RT-PCR assays could be enhanced by using other platforms or a modified protocol.

Several conclusions could be derived by comparing the results obtained with the monoplex and the duplex RT-PCR assays. Even though identical primers were used in both assays, the duplex RT-PCR assay was significantly ($P < 0.001$) more sensitive (Fig. 2 and Table 3). Several possibilities could explain these discrepant results. First, the two assays differed in respect to the extraction methods, RT-PCR conditions, and thermocycling conditions (annealing temperatures) used. For example, the higher annealing temperature used in the duplex RT-PCR assay (55°C) compared to that used in the swH1 monoplex RT-PCR assay (50°C) contributed to increased sensitivity (Fig. 3 and Table 3). Second, some of the discrepant results may simply reflect a Poisson distribution due to sampling error with low concentrations of template (11, 20). This phenomenon is almost impossible to control and is the most pronounced with low target concentrations, in which small changes in the amount of the nucleic acid template in a PCR mixture could generate a relatively large difference in the numbers of amplicons produced. A large number of replicates would be necessary to overcome this limitation. Similarly, PCR inhibitors are known to affect PCR amplification and could lead to considerable variations in the efficiency of the PCR. This hypothesis is highly plausible, since the monoplex and the duplex RT-PCR assays were performed by using different extraction methods; however, the lack of an exogenous internal

control prevents assessment of the contribution of PCR inhibitors. Further studies are being undertaken to combine the duplex RT-PCR assay with the detection of an internal control, such as bacteriophage MS2.

In addition to identifying influenza A virus, the duplex RT-PCR assay was highly sensitive at identifying swH1 (Table 3). During the validation period and retrospective analysis, 94% (62/66) and 92% (34/37) of the cases of pandemic (H1N1) 2009 influenza virus were found to be swH1 positive by the duplex RT-PCR assay. It should be noted that the annealing temperature of the duplex RT-PCR assay greatly influenced the ability of the assay to detect swH1 (Fig. 3 and Table 3). The use of an annealing temperature of 55°C rather than one of 50°C increased the number of swH1 targets detected (Table 3). Using this strategy, the duplex RT-PCR assay identified swH1 in the majority of cases of pandemic (H1N1) 2009 influenza virus, thereby considerably reducing the number of specimens forwarded to reference laboratories for confirmatory identification. However, we recognize there will be circumstances in which influenza A virus-positive specimens will have virus at amounts below the limit of detection of the swH1 primer pair. For the few cases that fit this criterion, low viral loads were observed by the real-time RT-PCR assay. Since the sensitivities of RT-PCR assays targeting influenza A virus are not equivalent (Table 3), confirmatory identification of pandemic (H1N1) 2009 influenza virus could be problematic when specimens contain low viral loads. The use of sequencing has been proposed by some to confirm the detection of pandemic (H1N1) 2009 influenza virus in clinical specimens; however, this methodology can also be problematic. Sequencing reactions use the amplicon generated from the RT-PCR assay as the template; therefore, amplicon contamination could result in false-positive sequence data. With the recognition that sequencing will always play an important role in the confirmatory identification of novel influenza viruses, other methods for the confirmatory identification of low-positive RT-PCR results should be sought (discussed below).

Since the RT-PCR assay optimal for the detection of pandemic (H1N1) 2009 influenza virus is unknown, a modified gold standard was used in this study to assess the clinical performance of all RT-PCR assays. A positive case was defined by the detection of concordant results between at least two RT-PCRs targeting different genomic regions and subsequent sequence analysis to ensure the specificities of the primers. As some discordant results between the various RT-PCR assays were observed, other experiments were required to ensure that the additional influenza A virus-positive cases detected could not be attributed to amplicon contamination. Specimens displaying discordant results were subjected to reextraction and repeat RT-PCR by the same duplex assay, a real-time swH1 RT-PCR assay, and reextraction by use of a larger volume of specimen (1 ml versus 140 μl) and a second RT-PCR with primers whose sequences were outside the original targeted region, followed by sequencing of the M gene. Using these strategies, all discordant results could be resolved. In light of all results, the duplex RT-PCR assay was deemed to be highly sensitive for the detection of influenza A virus and the confirmatory identification of pandemic (H1N1) 2009 influenza virus.

Until now, most infections attributed to pandemic (H1N1) 2009 influenza virus have been mild and self-limited. There is growing concern that this virus will evolve and lead to subsequent outbreaks of severe disease. Rapid detection of this novel influenza virus is paramount so that control measures may be implemented. We have shown that the duplex RT-PCR assay is a highly sensitive, accurate, and reliable method for the detection and confirmatory identification of pandemic (H1N1) 2009 influenza virus. While the real-time RT-PCR assay could permit a more timely diagnosis (12, 15, 22), not all laboratories have the infrastructure to offer such testing. The duplex RT-PCR assay is undoubtedly an attractive option for laboratories without this capability. Since the fate of this novel influenza virus is unclear, a conventional RT-PCR assay that uses generic reagents (without the need for probes or specialized kits) is ideal for any laboratory transitioning from low- to high-throughput screening for pandemic (H1N1) 2009 influenza virus. With generic reagents, supplies may also be more readily accessible as the global demand for testing peaks.

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